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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/US96/09616</p> <p>(22) International Filing Date: 7 June 1996 (07.06.96)</p> <p>(30) Priority Data:              08/486,821           7 June 1995 (07.06.95)           US              08/476,842           7 June 1995 (07.06.95)           US</p> <p>(71) Applicant (for all designated States except US): CERUS CORPORATION [US/US]; Suite 300, 2525 Stanwell Drive, Concord, CA 94520 (US).</p> <p>(72) Inventors; and          (75) Inventors/Applicants (for US only): COOK, David [US/US]; 3399 Freeman Road, Walnut Creek, CA 94595 (US). WOLLOWITZ, Susan [US/US]; 764 Beale Court, Walnut Creek, CA 94598 (US). NERIO, Aileen [US/US]; 1775 Ravizza Avenue, Santa Clara, CA 95051 (US).</p> <p>(74) Agent: WILKE, Kathryn, P.; Cerus Corporation, Suite 300, 2525 Stanwell Drive, Concord, CA 94520 (US).</p>		<p>(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b>  <i>With international search report.</i></p>
<p>(54) Title: TREATING RED BLOOD CELL SOLUTIONS WITH ANTI-VIRAL AGENTS</p> <p>(57) Abstract</p> <p>Methods and compositions for treating pathogens in material are described, including methods of decontaminating human fluids prior to processing in the clinical laboratory and methods for decontaminating blood products prior to <i>in vivo</i> use. The techniques handle large volumes of human blood products without destroying the function of the blood products. Novel compounds for photodecontaminating biological material are also contemplated which are compatible with clinical testing, in that they do not interfere with serum analytes.</p>		

## TREATING RED BLOOD CELL SOLUTIONS WITH ANTI-VIRAL AGENTS

This application is a continuation in part of U.S. Patent Application Serial  
5 No. 08/338,040, filed November 14, 1994.

### FIELD OF THE INVENTION

The invention generally relates to new compounds and methods for the  
*in vitro* inactivation of pathogens in biological material intended for *in vitro* or  
10 *in vivo* use, and in particular the inactivation of pathogens in solutions  
containing red blood cells, prior to clinical testing or transfusion.

### BACKGROUND

The presence of pathogens in blood products, as well as other biological  
15 materials, is recognized as a significant health problem to health workers as well  
as recipients of the materials.

With regard to health workers, a great volume of human fluids is handled  
daily as part of the routine monitoring of hospital patients by obtaining and  
testing human fluids (blood, urine, spinal fluid, etc.). Typically, each admitted  
20 patient has at least a tube of blood collected every day by a phlebotomist. During  
the transferring, portioning and testing process, each sample tube is handled by a  
clinical worker while its contents are exposed. This intensive handling of  
potentially infectious human fluids is not without health risk. The Occupational  
Safety and Health Administration (OSHA) estimates that over five million  
25 health workers, including hospital laboratory workers, are exposed to blood  
borne-pathogen infections in the work place annually. The pathogen responsible  
for the overwhelming majority of infections is the hepatitis B virus (HBV). The  
Center for Disease Control (CDC) estimates there are twelve thousand cases of  
HBV infection among health workers each year. Of these cases, over five  
30 hundred require hospitalization and approximately two hundred and fifty of  
these patients die (i.e. from fulminant hepatitis, cirrhosis or liver cancer). See  
Guidelines for Prevention of Transmission of HIV and HBV to Health-Care and  
Public Safety Workers, CDC (February 1989). Most full time laboratory employees  
contract hepatitis at least once during their career. Indeed, up to one third of all  
35 health care workers show serological evidence of a previous HBV infection. *Id.*

The red blood cell component is used primarily to treat trauma, chronic anemia, and blood loss due to surgery (particularly cardiac and liver surgery), including postoperative bleeding. D.M. Surgenor *et al.* Transfusion 32:458 (1992). Approximately twelve (12) million units of red cells are transfused into  
5 approximately four (4) million recipients annually in the United States alone. E.L. Wallace *et al.* Transfusion 33:139 (1993).

The safety of the blood supply cannot be assured by merely testing the blood for pathogens before transfusion. Most testing relies on the detection of antibodies to the pathogen in the prospective blood donor. It is now well-  
10 documented that infectious agents can be transmitted by "seronegative" blood donors, i.e. donors that have no detectable antibodies to the pathogen. For example, thirteen cases of transfusion-related AIDS have been reported to the Centers for Disease Control (CDC) among recipients of blood that was pre-tested and found negative for antibody to the HIV-1 virus.

Clerical errors and other mistakes further expose patients to contaminated, incorrectly tested or mislabeled blood. To complicate the problem, one bad unit can create several victims, since whole blood is routinely split into components. Mistakes are not infrequent in blood banks. Since the beginning of 1990, 29,586 blood bank errors and accidents have been reported to the FDA. "How Safe Is  
20 Our Blood," U.S. News and World Report, June 27, 1994, 68-78. Recalls by blood centers of blood released in error are generally ineffective because they take place months or years after the blood products have been transfused.

An alternative approach to eliminate transmission of diseases through blood products is to develop a means to inactivate pathogens in transfusion  
25 products. Some of these techniques such as heat [J. Hilfenhous *et al.* J. Biol. Std. 70:589 (1987)], solvent/detergent treatment [B. Horowitz *et al.* Transfusion 25:516 (1985)], gama-irradiation [G. Moroff *et al.* Transfusion 26:453 (1986)] or UV alone [K.N. Proudouz *et al.* Blood 70:589 (1987)] are completely incompatible with maintenance of red cell function.

Another means to inactivate pathogens is the use of methylene blue. S.J. Wagner *et al.* examined methylene blue as a virucidal for red cell solutions. S.J. Wagner *et al.* Transfusion 33:30 (1993). Photo treatment of red cells with methylene blue was found to cause loss of ATP, enhanced ion permeability, and binding of autologous immunoglobulin (IgG) to the red cell surface. It was  
35 speculated that some general (and undesirable) modification of the red cell membrane occurs as a result of the treatment.

product to a final concentration of the compound of between 1µg/ml and 250 µg/ml.

The present invention further contemplates the following independent embodiments: where the compound is added to the blood product to a final  
5 concentration of the compound of between 1µg/ml and 250 µg/ml; where the mixture is incubated for between 1 minute and 48 hours; where the mixture is incubated for between approximately 12 and 24 hours; where when the compound is added to the blood product, the compound is in a mixture comprising dextrose, sodium chloride, mannitol, adenine and H<sub>2</sub>O; where the  
10 method further comprising: transfusing the incubated mixture into a mammal; where the blood product comprises red blood cells; where the method further comprises washing the blood product to remove compound from the incubated mixture; where the pathogens comprise viral pathogens or bacterial pathogens; where the compound is selected from the group consisting of: 8-[3-(Bis-2-  
15 chloroethyl) amino]propyloxypsoralen, 5-[3-(Bis-2-chloroethyl) aminopropyloxy]methyl-8-methoxypsoralen, 5-[3-(Bis-2-chloroethyl) aminopropyloxy]methyl-8-methoxypsoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine; where  
20 more than one of the compounds is added to the blood product; where the method further comprises: removing the compound from the incubated mixture with an adsorbent material.

An alternative embodiment of the present invention comprises a method of inactivating pathogens in a blood product, comprising: adding a compound  
25 having a mustard group and a nucleic acid binding ligand, selected from the group consisting of a psoralen group and an acridine group, to a blood product comprising red blood cells suspected of containing pathogens, to create a mixture, the compound reaching a final concentration sufficient to inactivate substantially all of the pathogens, and incubating the mixture for between 1  
30 minute and 48 hours, without significant damage to the red blood cells, to create an incubated mixture.

The present invention further contemplates the following independent embodiments of this alternative embodiment: where the compound is added to the blood product to a final concentration of the compound of between 1µg/ml  
35 and 250 µg/ml; where when the compound is added to the blood product, the compound is in a solution comprising dextrose, sodium chloride, mannitol,

method further comprises removing the compound from the incubated mixture with an adsorbent material.

The present invention contemplates additionally an improved method of treating clinical samples, comprising, in the following order: providing a  
5 compound having a mustard group and a nucleic acid binding ligand, selected from the group consisting of a psoralen group and an acridine group, and a clinical sample intended for *in vitro* clinical testing; adding the compound to the clinical sample, to create a mixture, incubating the mixture for between 1 minute and 48 hours, and measuring the level of a clinical chemistry analyte in  
10 the clinical sample.

The present invention further contemplates the following independent embodiments of the improved method: where the compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxypsoralen, 5-[3-(Bis-2-chloroethyl) aminopropyloxy]methyl-8-methoxypsoralen, 5-[3-(Bis-2-  
15 chloroethyl) aminopropyloxy]methyl-8-methoxypsoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine; where the clinical sample comprises red blood cells; where the red blood cells further comprise viral pathogens or bacterial pathogens; where the incubation step is  
20 performed without significant damage to the clinical chemistry analog.

The present invention contemplates a method of inactivating pathogens in a red blood cell containing composition, comprising: adding a compound having a nucleic acid binding ligand and a mustard group to a blood product comprising red blood cells, where the blood product is suspected of containing  
25 pathogens, to create a mixture, the compound reaching a final concentration sufficient to inactivate substantially all of the pathogens, incubating the mixture *in vitro* for between 1 minute and 48 hours while retaining the ability of the blood product to function for the purpose the blood product was prepared, to create an incubated mixture, and transfusing the incubated mixture into a  
30 mammal.

The present invention further contemplates the following independent embodiments of the improved method: where the compound is added to the blood product to a final concentration of the compound of between 1µg/ml and 250 µg/ml; where when the compound having a nucleic acid binding ligand and  
35 a mustard group is added to the blood product, the compound is in a solution comprising dextrose, sodium chloride, mannitol, adenine and H<sub>2</sub>O; where the

chloroethyl)aminobutoxy)methyl-4,5',8-trimethylpsoralen; and, 5-[N,N-bis(2-chloroethyl)amino)methyl-8-methoxypsoralen. It is contemplated that in various embodiments of the present invention the treated blood product or biological sample may be mixed with the compound prior to or during incubation.

#### DESCRIPTION OF THE FIGURES

FIG. 1 is a graph showing reduction in titer of R17 treated with varying concentrations of quinacrine mustard in either Adsol or dimethyl sulphoxide (DMSO). The horizontal dotted line represents the limit of detection of the assay used.

FIG. 2 is a graph showing inactivation of R17 by quinacrine mustard.

FIG. 3 is a graph showing the inactivation kinetics of quinacrine mustard.

FIG. 4 is a graph showing the reduction in titer of R17 as a function of time of incubation of quinacrine mustard in Adsol.

FIG. 5 is a graph showing the reduction in R17 inactivation activity as a function of time when incubated in the presence of either Adsol, red blood cells or Amberlite XAD-16™.

FIG. 6 is a graph showing the effects of quinacrine mustard at varying concentrations on extra-cellular potassium levels.

FIG. 7 is a graph showing the reduction in titer of R17 in packed red blood cells treated with varying concentrations of quinacrine mustard; the horizontal dotted line represents the limit of detection of the assay used.

FIG. 8 is a graph showing the activity of quinacrine mustard, after incubation in red blood cells, with or without the presence of Amberlite XAD-16™, in an Ames assay using strain TA 1537.

FIG. 9 is a graph showing the inactivation of a bacterial strain, *Staphylococcus Epidermis*, using quinacrine mustard at varying concentrations.

#### DESCRIPTION OF THE INVENTION

The present invention generally relates to new compounds and methods for the *in vitro* inactivation of pathogens in biological material intended for *in vitro* or *in vivo* use, and in particular the inactivation of pathogens in solutions containing red blood cells, prior to clinical testing or transfusion. In accordance with the present invention, a compound having a nucleic acid binding ligand and a mustard group is selectively employed to treat contamination by nucleic acid-containing microorganisms, including pathogenic viruses and bacteria.

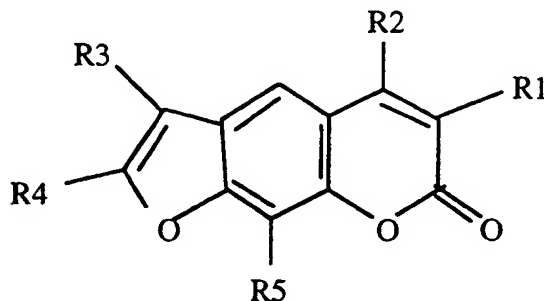
acriflavine, actinomycins, anthracyclines, beta-rhodomyacin A, daunomycin, thioxanthones, miracil D, anthramycin, mitomycin, echinomycin, quinomycin, triostin, diacridines, ellipticine (including dimers, trimers and analogs), norphilin A, fluorenes and fluorenones, fluorenodiamines, quinacrine, benzacridines, phenazines, phenanthradines, phenothiazines, chlorpromazine, phenoxazines, benzothiazoles, xanthenes and thio-xanthenes, anthraquinones, anthrapyrazoles, benzothiopyranoindoles, 3,4-benzpyrene, benzopyrene diol epoxide, 1-pyrenyloxirane, benzanthracene-5,6-oxide, benzodipyrones, benzothiazoles, quinolones, chloroquine, quinine, phenylquinoline carboxamides, furocoumarins, such as psoralens and isopsoralens, ethidium salts, propidium, coralyne, ellipticine cation and derivatives, polycyclic hydrocarbons and their oxirane derivatives, and echinomycin; b) minor groove binders such as distamycin, mitomycin, netropsin, other lexitropsins, Hoechst 33258 and other Hoechst dyes, DAPI (4',6'-diamidine-2-phenylindole), berenil, and triarylmethane dyes; c) major groove binders such as aflatoxins; d) molecules that bind by electrostatics (phosphate backbone binders), such as spermine, spermidine, and other polyamines; e) nucleic acids or analogues which bind by such sequence specific interactions as triple helix formation, D-loop formation, and direct base pairing to single stranded targets.

While not limited to any particular mechanism, it is believed that the nucleic acid binding ligand functions as a carrier (or anchor) that targets (or directs) the molecule to nucleic acid, interacting non-covalently therewith.

#### 1. Psoralens as Noncovalent Nucleic Acid Binding Groups

The present invention contemplates a specific class of compounds which use a psoralen group as a nucleic acid binding group. These compounds are particularly suitable for use in the present invention. Previous nucleic acid specific alkylating agents typically contain an alkylating moiety such as a chloroethylamine fragment, connected to a nucleic acid specific group, an intercalator (e.g., acridine), or a minor groove binder. These moieties are mutagenic in themselves. After the residual alkylating agent has been hydrolysed from the compound, the residue may still be rather mutagenic. In contrast, compounds having a psoralen nucleic acid binding group display substantially reduced mutagenicity, thus providing an improved safety factor. Psoralens are well known as nucleic acid intercalators but their utility has mainly been as photoactive agents which covalently bind to the nucleic acids upon irradiation

The compounds may be neutral amines, or their salts.



Ring construction of psoralens, and their functionalization with displaceable groups, X, (where X = Cl, Br, I, OSO<sub>2</sub>CH<sub>3</sub>, etc.) is described in the literature (Hearst et al, US patents 4,124,598; 4,196,281; Kaufman, US patents 4,269,851; 4,269,852; 4,294,822; 4,298,614; 4,370,344; Wollowitz et al., US patent 5,399,719; Antonello, S. C., et al., *Farmaco* (1978) 34, 139).

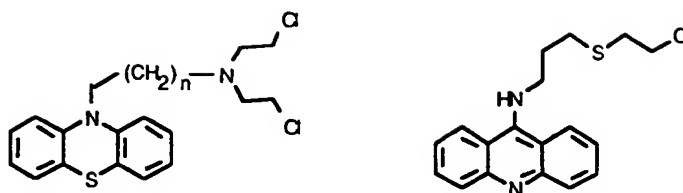
The desired products are constructed by one of three routes. In the first, a 2-hydroxyethylamine (e.g., diethanolamine) is reacted directly with psoralen-(CH<sub>2</sub>)<sub>n</sub>X where X is a readily displaceable group such as a halide, mesylate or tosylate. The chain is attached to the psoralen at the 3,4,4',5', or 8 positions, other substituents may be on the psoralen ring, and n = 1-6. In a second steps, the hydroxy groups of the intermediate are then converted to chloro or bromo groups by standard means, for example with thionyl chloride to give the desired product.

In the second route, the functionalized psoralen is reacted with HY-(CH<sub>2</sub>)<sub>m</sub>-OH, where Y = NH, S, O and m = 2-6). The terminal alcohol is then converted to a readily displaceable group (halo, mesylate, etc.) by standard means, then reacted with the (2-hydroxyethyl)amine. The resultant compound is converted into a haloethylamine-functionalized product as described above.

In the final route, the HY-(CH<sub>2</sub>)<sub>m</sub>-N(CH<sub>2</sub>CH<sub>2</sub>OH)<sub>2</sub>, where m = 2 to 6, is prepared as described in the literature (e.g., Peck, R. M., Preston, R. K., Creech, H. J., *J. Amer. Chem. Soc.*, (1959) 81, 3984), and reacted directly with the functionalized psoralen. Again, conversion of the hydroxy groups to halides gives the desired psoralen mustard products.

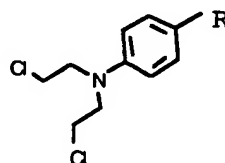
#### B. Mustard Group

The second characteristic that compounds of the present invention have in common is that they contain at least one mustard group. A "mustard group"

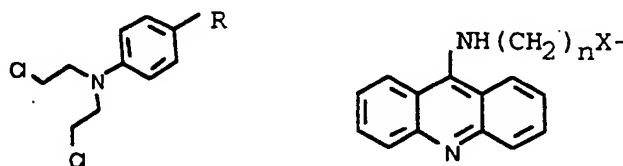


Nitrogen mustards are thoroughly described in the literature. E.g., see Gravatt, G.L., et al., "DNA-Directed Alkylating Agents. 4. 4-Anilinoquinoline-Based Minor Groove Directed Aniline Mustards," J. Med. Chem. 34:1552 (1991);  
 5 Cummings, J., et al., "Determination of Reactive Nitrogen Mustard Anticancer Drugs in Plasma by High-Performance Liquid Chromatography Using Derivatization," Anal. Chem. 63:1514 (1991). They are known to be potent  
 10 alkylators of nucleic acid and due to this mode of action, they have been widely studied as anti-tumor agents. Several have found practical use in the clinic (e.g. aniline mustard, chlorambucil, melphalan).

One class of nitrogen mustards is the aniline mustard class. These compounds have at least one haloethylaminoaniline group, where the haloethyl may be mono or bis. An example of a bis(haloethyl)aminoaniline group appears  
 15 below (where R is the point of linkage to other groups):



A specific aniline mustard group is the acridine carried aniline mustards (described in Gravatt, et al., J. Med. Chem. 34:1552), where R comprises a linking  
 20 group (for example O, CH<sub>2</sub>, S, COHN, or CO, however, other linking groups are contemplated) which links the mustard group to a second component, an acridine group. An example of the components of a 9-aminoacridine carried aniline mustard appears below (where X is the linking group):



blood and blood components, such as urine, sputum, feces, spinal fluid, and other materials removed from mammals for clinical testing.

5

### III. INACTIVATION OF PATHOGENS

The present invention contemplates treating a blood product with a compound having a nucleic acid binding ligand and a mustard group to  
10 inactivate contaminating pathogen nucleic acid sequences before using the blood product.

#### A. Inactivation In General

The term "inactivation" is here defined as the altering of the nucleic acid  
15 of a unit of pathogen so as to render the unit of pathogen incapable of replication. This is distinct from "total inactivation", where all pathogen units present in a given sample are rendered incapable of replication, or "substantial inactivation," where most of the pathogen units present are rendered incapable of replication. "Inactivation efficiency" of a compound is defined as the level of  
20 inactivation the compound can achieve at a given concentration of compound. For example, if 100  $\mu$ M of a hypothetical compound X inactivated 5 logs of HIV virus whereas under the same experimental conditions, the same concentration of compound Y inactivated only 1 log of virus, then compound X would have a better "inactivation efficiency" than compound Y.

25 To appreciate that an "inactivation" method may or may not achieve "total inactivation," it is useful to consider a specific example. A bacterial culture is the to be inactivated if an aliquot of the culture, when transferred to a fresh culture plate and permitted to grow, is undetectable after a certain time period. A minimal number of viable bacteria must be applied to the plate for a signal to be  
30 detectable. With the optimum detection method, this minimal number is 1 bacterial cell. With a sub optimal detection method, the minimal number of bacterial cells applied so that a signal is observed may be much greater than 1. The detection method determines a "threshold" below which the "inactivation method" appears to be completely effective (and above which "inactivation" is,  
35 in fact, only partially effective).

bacteria, protozoa, and viruses as well. It is not intended that the present invention be limited by the number or nature of pathogens inactivated. Importantly, however, the treatment of the present invention has been found to block the replication of the HIV virus. Had an effective decontamination method been available prior to the advent of the AIDS pandemic, no transfusion associated HIV transmission would have occurred. Decontamination based on compounds having a nucleic acid binding ligand and a mustard group has the potential to eliminate all infectious agents from the blood supply, regardless of the pathogen involved.

#### C. Selecting Compounds for Inactivation of Pathogens

In order to evaluate a compound to decide if it would be useful in the decontamination methods of the present invention, two important properties should be considered: 1) the compound's ability to inactivate pathogens, 2) its mutagenicity after treatment, and 3) the ability of the treatment to perform steps 1) and 2) while retaining the ability of said blood product to function for the purpose said blood product was prepared. The ability of a compound to inactivate pathogens may be determined by several methods. One technique is to perform a bacteriophage screen, an assay which determines nucleic acid binding of test compounds. A screen of this type, an R17 screen, is described in detail in an example, below. If the R17 screen shows inactivation activity, it is useful to directly test the compound's ability to inactivate a virus. One method of performing a direct viral inactivation screen is described in detail in an example below for cell free HIV.

The R17 bacteriophage screen is believed to be predictive of HIV inactivation efficiency, as well as the efficiency of compounds against many other viruses. R17 was chosen because it was expected to be a very difficult pathogen to inactivate. It is a small, single stranded RNA phage. Without intending to be limited to any means by which the present invention operates, it is expected that shorter pieces of nucleic acid are harder to inactivate because they provide a smaller target for the compound. Thus it is expected that under conditions that result in the inactivation of R17 the inactivation of many viruses and bacteria will also be obtained.

The cell free HIV screen complements the R17 screen by affirming that a given compound which has tested positive in R17 will actually work effectively

to be used *in vivo*, it is then taken through Step III. A biological material decontaminated by a method of the present invention is screened in the Ames assay to determine whether any compound that remains after decontamination is mutagenic. Finally, if the residual material does not show significant  
 5 mutagenicity in the Ames assay, the compound is identified as a useful agent for inactivation of pathogens in products to be used *in vivo* as well.

TABLE 1

STEP	SCREEN	RESULT	INTERPRETATION
I	R17	>1 log kill by any concentration	potential compound, go to step 2
		<1 log kill	compound is ineffective as an inactivation treatment
II	Viral Inactivation	> 1 log kill by any concentration	useful for clinical sample decontamination go to step 3
		< 1 log kill	compound is ineffective as an inactivation treatment
III	Ames	less mutagenic than AMT	useful agent for inactivation

10 The third property, retaining the ability of said blood product to function for the purpose said blood product was prepared, can be tested as follows. The treated blood product can be screened in one or more tests for blood product function that are generally acceptable. If a test sample is treated, screened and compared to a control sample, a determination can easily be made whether the  
 15 variation, if any from the control sample, are within an acceptable range according to current standards of blood banking practice. For example, to screen a particular compound for use in a method of the present invention to treat red blood cell concentrates, one can determine whether the treated red blood cells retain the ability to function for the purpose they are prepared by measuring the stimulation of IgG binding in the agglutination experiment described in Example  
 20 10.

By following these instructions, a person can determine which compounds would be appropriate for use in methods of the present invention.

protein, albumin, total globulin, albumin/globulin ratio, billirubin, alkaline phosphatase, lactate dehydrogenase, glutamate transferase, aspartate transaminase, alanine aminotransferase, uric acid, iron, triglycerides, and cholesterol.

5 In the decontamination of clinical samples, the goal is to decontaminate the sample so that infectious agents cannot be transferred to clinical laboratory workers. Because the samples will not be transfused into a recipient, there is less concern that residual compound be removed from the sample. Thus removal techniques may not be desired. The present invention contemplates that the  
10 compound may be in the clinical sample test tube prior to drawing the sample from the patient, or it may be added after drawing. Once the compound has contacted the sample, the sample preferably is thoroughly mixed, then incubated. The sample may then be screened in the desired panel of clinical chemistry tests without concern for spreading infectious diseases.

15

## 2. Decontamination of Blood Products for Transfusion.

The compound for decontamination may be introduced to the whole blood prior to fractionating, by adding to the blood bag before or after blood is drawn. Alternatively, the compound may be added after fractionation of the  
20 blood, decontaminating the individual fractions.

For the decontamination of particularly viscous blood products, such as red blood cell concentrates, active mixing of the blood product with the compound may be desirable to achieve rapid and complete distribution of the compound. The present invention contemplates embodiments in which a  
25 compound of the present invention is actively mixed with the blood product to be treated prior to or during the incubation of the blood product with the compound. The following is a non-exclusive list of examples that the present invention contemplates as appropriate technologies for mixing: A flow system of mixing compounds with blood products is contemplated where standard  
30 tubing fitting shapes such as T or Y shapes are used to provide orifices for enhanced mixing. These shapes may easily be optimized to enhance turbulence or create a swirling pattern at the point of mixing. One embodiment contemplates changing the direction of one or more flow streams for more energetic mixing. Concentric or annular flow streams are also contemplated.  
35 A static mixer with pins and vanes is also contemplated to enhance mixing.

the present invention. Blood separation techniques which are contemplated as appropriate technologies include those used in treatments such as blood-oxygenation, plasmapheresis, leukopheresis, hemopheresis, extracorporeal chemotherapy, hyperthermia, hypothermia, bone marrow transfusions, blood transfusions processing by heart lung machines during surgery and dialysis for patients with kidney failure. There follows a list of separating technologies that may be applied in the present invention to wash blood products. These are presented as examples, and all patents and publications listed are hereby incorporated by reference. This list is not to be considered as limiting the invention in any way, but is merely presented for guidance.

Field Flow Fractionating (FFF), is a chromatographic method for fractionating macromolecules and fine particles. A field acts perpendicular to flow through a narrow channel, forcing particles toward a wall. Simultaneously, diffusion tends to re disperse the particles. The particles least affected by the field, and most dispersed by diffusion in the cross section move downstream most quickly. A pulse of a mixture dispersed or dissolved in a fluid carrier which flows steadily through a tube will thus emerge in several fractions or peaks, as in chromatography. Various types of external fields have been employed, yielding the following FFF subclasses: sedimentation (centrifugal), thermal, thermogravitational, flow (pressure gradient causes cross flow through membrane walls), and concentration. The descriptions hereunder specifically address the following separation technologies: spinning membrane techniques, hollow fiber separators, centrifugal separation devices, separation techniques using flat membranes with tangential flow, and passive filtration techniques relying on gravity flow to pass through a filter.

There are numerous membrane-moderated separation devices and methods known in the art. For example, U.S. Pat. No. 4,375,414 discloses immobilizing immunoactive materials on the side of a membrane on which the fluid being treated is flowing or having the material carried in fluid on the opposite side of the membrane. U.S. Pat. No. 4,266,026 disclose use of an anisotropic membrane. U.S. Pat. No. 4,191,182 to Popovich, et al. describes a method and apparatus for plasmapheresis. Again, the system described employs a membrane with the appropriate pore sized to fractionate blood into cellular and plasma components.

A variety of spinning membrane techniques have been detailed in the literature. One such technique is embodied in the Autopheresis C plasma

### Monitoring the Removal Process

The fluorescent characteristics of some compounds of the present invention may be used to monitor the removal process. Measurement of fluorescence provides the opportunity for every treated sample to have a quality control check prior to transfusion to confirm treatment performance and completion. The present invention contemplates that an actinic light source with a low-pass filter <420 nm and a photodiode detector with a high pass filter >450 nm be placed in the waste stream so as to measure fluorescence. Fluorescence measurement is a reproducible way of determining whether the removal process is complete.

### 3. Decontamination of Vaccines and Other Biological Compositions

Vaccines and other biological compositions which are not derived from blood, such as recombinant DNA produced proteins and oligopeptide ligands, may also be decontaminated using methods of the present invention. Recombinant DNA produced proteins often are manufactured in large quantities in host organisms. Introduction of the decontamination compound may occur prior to amplification, so that as the host organisms grow, the compound is incorporated into the organism. Alternatively, the compound may be added after manufacture, but before the product is introduced into a mammal.

Removal of the compound before use may be desired here as well as with blood products for transfusion. Those methods mentioned above apply equally well in the case of vaccines and other biological compositions.

### V. PRESERVATION OF BIOCHEMICAL PROPERTIES OF TREATED MATERIAL

When treating blood products to be used *in vivo*, one must ask whether the process or the compounds used alter the *in vivo* activity of the treated material. For example, red blood cell transfusion is a well established efficacious treatment for patients suffering large blood loss. However, if the inactivation treatment used greatly reduces the *in vivo* life of the red blood cells, then the treatment has no practical value. The compounds of the present invention are useful in inactivation procedures because the reaction can be carried out at temperatures compatible with retaining biochemical properties of blood and blood products. But not all methods of pathogen inactivation will inactivate

for producing a vaccine for inoculation of a mammalian host susceptible to infection by a virus comprises growing culture of virus, isolated from an infected host, in a suitable mammalian cell culture, exposing at least one of the seed viruses to a compound having a nucleic acid binding ligand and a mustard  
5 group for a time sufficient to inactivate the virus to a non-infectious degree, under conditions which substantially preserve the antigenic characteristics of the inactivated viral particles, and combining said inactivated virus with a suitable adjuvant.

The inactivated virus may be formulated in a variety of ways for use as a  
10 vaccine. The concentration of the virus will generally be from about  $10^6$  to  $10^9$  plaque forming units (pfu)/ml, as determined prior to inactivation, with a total dosage of at least  $10^5$  plaque forming units per dose (pfu/dose), usually at least  $10^6$  pfu/dose, preferably at least  $10^7$  pfu/dose. The total dosage will usually be at or near about  $10^9$  pfu/dose, more usually being about  $10^8$  pfu/dose. The vaccine  
15 may include cells or may be cell-free. It may be an inert physiologically acceptable medium, such as ionized water, phosphate-buffered saline, saline, or the like, or may be administered in combination with a physiologically acceptable immunologic adjuvant, including but not limited to mineral oils, vegetable oils, mineral salts, and immunopotentiators, such as muramyl dipeptide. The  
20 vaccine may be administered subcutaneously, intramuscularly, intraperitoneally, orally, or nasally. Usually, a specific dosage at a specific site will range from about 0.1 ml to 4 ml, where the total dosage will range from about 0.5 ml to 8 ml. The number of injections and their temporal spacing may be highly variable, but usually 1 to 3 injections at 1, 2 or 3 week intervals are effective.

25

### EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

30 In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar);  $\mu$ M (micromolar); N (Normal); mol (moles); mmol (millimoles);  $\mu$ mol (micromoles); nmol (nanomoles); gm (grams); mg (milligrams);  $\mu$ g (micrograms); L (liters); ml (milliliters);  $\mu$ l (microliters); cm (centimeters); mm (millimeters);  $\mu$ m (micrometers); nm (nanometers);  $^{\circ}$ C  
35 (degrees Centigrade); HPLC (High Pressure Liquid Chromatography); Q (quinacrine); QM (quinacrine mustard); DMSO (dimethylsulfoxide); Htc

## EXAMPLE 1

This example measures the R17 inactivation activity of quinacrine mustard (QM) solutions made in either Adsol or DMSO. The bacteriophage R17 has a single stranded RNA genome of approximately  $1.2 \times 10^6$  daltons, and is  
5 difficult to inactivate compared to many other targets. See generally L. Lin *et al.*, Blood 74:517 (1989). The advantage of the R17 system is that inactivation can be readily assayed in the laboratory.

The assay used to determine inactivation measures the ability of the phage to subsequently infect bacteria and inhibit their growth. The phage was grown  
10 up in Hrf 3000 bacteria. (R17 and Hrf 3000 were obtained from American Tissue Culture Collection (ATCC), Washington, D.C.). First, the R17 stock virus was diluted ( $10.9 \log_{10}$ /ml in LB broth) 1:20 in Adsol (R17-Adsol). Then a 30% hematocrit (Htc) red blood cell concentrate in R17-Adsol mixture was prepared by  
15 spinning down red blood cells (RBC) from whole blood and resuspending 3.5 ml RBC pellet in 7.0 ml R17-Adsol. In this, and the following experiments, Htc was measured on a Model F800 Sysmex cell counter (Toa Medical Electronics, Kobe, Japan). Ten 1 ml aliquots of the samples were then transferred to sterile tubes.

Approximately 2 mg of QM, commercially available from Aldrich, Inc., Milwaukee, WI, was weighed out into each of two tubes. Samples were then  
20 dissolved in DMSO or Adsol, respectively, to a final concentration of 0.4 mg/ml. QM in Adsol is a suspension, not a solution, at this concentration.

Next, the QM suspension was added to the R17-Adsol samples to achieve the following final concentrations of QM in the sample tubes: 2.5, 5.0, 10, or 20  $\mu\text{g}/\text{ml}$ . The QM was completely solubilized at these concentrations. Positive  
25 control samples were also prepared, where 50  $\mu\text{l}$  of either Adsol or DMSO was added to R17-Adsol samples. The samples were allowed to stand at room temperature for at least 1 hour. Then the samples were titered by an R17 phage assay. Sterile 13 ml dilution tubes were prepared with LB broth. To make the  
30 dilutions, a 0.1 ml aliquot of the solution of phage was added to the first dilution tube of 0.4 ml of media. Then 0.02 ml of this solution was added to the second tube of 0.5 ml media (1:25). The second solution was then diluted serially (1:25) into the remaining tubes. To each diluted sample was added 0.05 ml of Hrf 3000 bacteria cultured overnight and 3 ml of molten LB top agar. The mixed materials  
35 were poured onto LB broth plates. After the top agar hardened, the plates were incubated at  $37^\circ\text{C}$  overnight. Plaques were counted the following morning and

QM. The samples were then titrated in an R17 phage assay, as described in Example 1, above.

The results are shown in Table 3 and FIG. 2. It is clear from the data that QM inactivates R17 in all of the Htc tested.

5

TABLE 3

Sample #	Htc (%)	QM ( $\mu\text{g/ml}$ )	Log Titer
1	0	0	8.8
9	0	1.0	2.0
10	1	1.0	4.0
11	3	1.0	2.1
12	10	1.0	2.6
13	30	1.0	2.4

Another experiment was performed to test the inactivation ability of a novel compound, Compound 1. A 1:1000 dilution of R17 (stock titer was 11.9 logs) was prepared in 25 ml packed red blood cells. To each of 5 tubes was added 5 ml of this R17-packed red blood cell solution. Compound 1 was then dissolved in saline to a final concentration of 3 mg/ml. The compound in solution was added to the 4 tubes as follows: the first tube, the control tube, received saline only; the second tube received 10  $\mu\text{g/ml}$  of Compound 1 in saline; the third tube received 30  $\mu\text{g/ml}$  of Compound 1 in saline; the forth tube received 100  $\mu\text{g/ml}$  Compound 1 in saline and the final tube received 300  $\mu\text{g/ml}$  of Compound 1 in saline. The tubes were mixed and then incubated at 4°C overnight. The results showed R17 inactivation activity. Concentrations above 30  $\mu\text{g/ml}$  inactivated approximately 4 logs of R17 with a starting titer of 10 logs of R17.

20

## EXAMPLE 3

This example sets forth the kinetics of R17 inactivation by QM. To measure the kinetics of inactivation, reactive QM must be quenched so that intermediate time points provide a reliable measure of the R17 inactivation at a particular time. Two methods were used here in combination to quench the reaction. First, NAC was added to samples to react with excess QM. Second, samples were rapidly diluted into LB medium to reduce the effective QM concentration in the sample. The control experiments described below

25

(250 µl) was added to quenching tube 13. Then samples 9 and 11 were diluted into LB broth for phage assay.

The experimental samples were treated next. Phage (1.0 ml) was removed into a sterile 15 ml tube. QM (1.0 ml, 1.0 µg/ml) was added. This mixture was removed (by 200 µl aliquots) into quenching tubes 1-8 at the following times: 0, 2, 4, 8, 16, 32, 64, and 128 minutes. The samples were mixed and immediately diluted into LB broth for phage assay. Finally, samples 10, 12, and 13 were diluted into LB broth for phage assay.

Results are shown in Table 5 and FIG. 3. While NAC alone does not kill R17 (compare samples #11 and #12 with sample #13), when added before QM, NAC provided a substantial, but not complete protection against QM inactivation (compare samples #7 and #10). The combination of NAC and dilution resulted in almost complete quenching of QM activity (compare samples #1 and #13). QM inactivation of R17 was complete within 2 hours.

TABLE 5

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13
Log Titer	8.11	6.81	5.79	4.10	3.74	2.69	2.39	≤2.4	8.66	6.32	8.73	8.35	8.80

## EXAMPLE 4

This experiment measures the loss of QM activity upon pre-incubation of drug in Adsol. It is believed that mustards react by thermally allowed pathways. They can be hydrolyzed in aqueous solution. This experiment was designed to measure loss of QM anti-viral activity in a particular aqueous solution, Adsol. Previous results have shown that QM anti-viral activity did not decrease rapidly upon pre-incubation of the drug in Adsol. (Results not shown). A concern in those experiments was the possibility of light-dependent inactivation, because samples were diluted into LB without making extraordinary efforts to shield ambient light, and because acridines are known to inactivate by photodynamic effects. This experiment was repeated under conditions where ambient light levels were carefully controlled throughout the experiment, in order to exclude the possibility that R17 inactivation was due to light-mediated effects. Also, additional controls were added to examine the effects of light in samples that were deliberately exposed to room lights and to examine the inactivation by the parent compound, quinacrine, the structure of which follows:

## EXAMPLE 5

QM activity was not diminished after a 4 hour pre-incubation in Adsol, as shown by Example 4, above. A goal of this example is to determine whether QM is inactivated more rapidly by pre-incubation in the presence of red cells. This example also examines the kinetics of removal of QM from red blood cell solutions by an adsorbent material, to establish the effectiveness of a removal technique in removing compounds containing a mustard group.

First, phage dilutions were prepared. R17 (11.3 log/ml stock) was diluted 1:10 into Adsol: 0.7 ml phage + 6.3 ml Adsol. Diluted phage (0.5 ml) was placed into 15 sterile 1.5 ml tubes labeled 1-15. The treatment for each tube is shown in Table 7.

TABLE 7

Sample #	Treatment	Time (min)
1	none	--
2	QM-adsol	0
3	QM-adsol	240
4	QM-RBC	0
5	QM-RBC	15
6	QM-RBC	30
7	QM-RBC	60
8	QM-RBC	120
9	QM-RBC	240
10	QM-XAD	0
11	QM-XAD	15
12	QM-XAD	30
13	QM-XAD	60
14	QM-XAD	120
15	QM-XAD	240

Next, QM solutions were prepared. Approximately 20 ml of packed red blood cells (PRBC) were spun down in a 50 ml conical tube at 1600 rpm for 9 minutes. The volume of the pellet after spinning was 17 ml. Approximately 3 mg QM was weighed out on a weighing paper in a biosafety cabinet (actual weight was 4.5 mg). The sample was then transferred to a 50 ml conical tube. The sample was

The results appear in Table 8, above, and FIG. 5. QM anti-viral activity was removed upon a 4 hour pre-incubation with red blood cells. The adsorbent removal material, Amberlite XAD-16™, also removed QM from blood within 1 hour. These results suggest that either incubation in the presence of red blood cells or treatment with an adsorbent resin, or the two treatments combined, will be sufficient to rapidly remove residual QM after inactivation.

#### EXAMPLE 6

10 The purpose of this example is to measure inactivation of duck hepatitis B virus (DHBV) by a method of the present invention. DHBV was chosen as a model for human hepatitis B virus because of the similarities in design between the two viruses. See Ganem, D. and Varmus, H. "The Molecular Biology of the Hepatitis B Viruses," Ann. Rev. Biochem. 56:651 (1987).

15 Infected duck hepatocytes were prepared as follows. Duck hepatocytes were isolated from the livers of approximately 1 week old ducklings. Ducklings were prescreened and found negative for DHBV. Each of the ducklings was anesthetized, then infused with 0.5 ml sodium heparin via the portal vein. Next, each duckling was perfused with 75 ml of a solution containing 200 ml 1X MEM/Earle's BSS + 2 ml Hepes buffer + 2 ml of 1% EGTA (in 1X MEM). Then,  
20 the ducklings were perfused for 20 minutes with a filter sterilized solution containing 30 mg of Collagenase A (commercially available from Boehringer-Mannheim Biochem., Indianapolis, IN) + 200 ml Ham's F-12/DMEM medium.

At this point, the liver was removed, cut up into a fine mush and placed  
25 in a 125 ml bottle containing 50 ml Ham's F-12/DMEM. Approximately 10 ml of a solution containing 5 mg DNase I and 25 ml Ham's F-12/DMEM was added to the liver suspension. The suspension was spun at 200 rpm for 10 minutes.

The suspension was then strained through gauze pads, the 125 ml bottle was rinsed with the remaining 15 ml of the DNase I solution and the rinsing was  
30 also strained into the liver suspension. The cell suspension was equally divided into 2 x 50 ml centrifuge tubes and pelleted at 50 x g for 2 minutes. The pellets were resuspended in 10 ml of a solution containing Medium 199/Earle's BSS, 5% calf serum and pelleted. This process was repeated two more times. The third pelleting was resuspended in 10 ml plating medium. Another 10 ml plating  
35 medium was added to each tube.

then analyzed by PCR and by slot blot hybridization to confirm the presence of viral DNA.

Slot blot hybridization was performed for all of the samples after harvesting DNA from tissue culture samples. PCR analysis was performed on selected samples. Samples were denatured with 3M NaOH, as were plasmid pD1.5G DNA samples for labeling. Samples were then neutralized with NH<sub>4</sub>OAc. 400 µl of 1M NH<sub>4</sub>OAc was added to each well of a Mini Fold II Slot Blot Apparatus, commercially available from VWR Scientific, Greenbelt, MO, fitted with a filter, as were aliquots of each sample. Vacuum was applied to the apparatus until all samples had been pulled through the filter. The filter was then baked to dry. Next, the filter was pre-hybridized in a mixture of 250 ml of 20X SSC (175.3 g NaCl, 88.2 g Na citrate in 800 ml H<sub>2</sub>O), 50 ml of 50X Denhardt's solution (5g Ficoll, available from Sigma, St. Louis, MO, 5g polyvinylpyrrolidone, and 5 g bovine serum albumin with 500 ml H<sub>2</sub>O), 20 ml of mg/ml denatured salmon sperm DNA, 180 ml H<sub>2</sub>O, 500 ml formamide and 10 ml of 10% solution of sodium dodecyl sulfate in H<sub>2</sub>O. Probe was prepared as follows: 3 µl of pD1.5G (67 ng/µl) and 5 µl of 15 ng/µl random hexamer oligonucleotides were heated and cooled again, then 4 µl of 5X labeling buffer, 2 µl of dGAT mixture (5 mM each of dGTP, dATP, dTTP, in TE), 1 µl of Klenow, and 5 µl of [<sup>32</sup>P]dCTP was added and incubated. Reaction was stopped by adding 25 mM EDTA. Then 5 X 10<sup>5</sup> counts per minute of probe per ml of hybridization solution was added to the filter and allowed to hybridize overnight. The filter was removed, and low stringency wash solution (50 ml of 20X SSC, 940 ml of H<sub>2</sub>O, and 10 ml of 10% SDS) was added to cover the filter for a wash during shaking, which was repeated 2 times, the last time adding high stringency was solution (5 ml of 20X SSC, 990 ml of H<sub>2</sub>O, and 10 ml of 10% SDS) instead. The filter was then exposed to film to obtain an appropriate exposure, and the film was then scored for positive hybridization. A negative control sample containing calf thymus DNA was also run. Table 10 summarizes PCR and slot blot hybridization data. (NP signifies that PCR was "not performed" for that sample. A plus sign signifies that DHBV nucleic acid was amplified in PCR. A minus sign signifies that it was not amplified).

TABLE 10

Sample #	Incubation (days)	Plate #'s	Blot Results	PCR Results
1	10	1*,2	-,-	-
2	10	3,4	-,-	NP
2	15	5*,6	-,-	-
3	10	7,8	-,-	NP
3	15	9*,10	-,-	+
4	10	11*,12	-,-	-
4	15	13*,14	±,+	+
5	10	15*,16*	+, -	+, +
5	15	17*,18	+,+	+
6	10	19,20*	+,+	+
6	15	21*,22	+,+	+
7	10	23,24	-,-	NP
7	15	25,26	-,-	NP
8	10	27,28	-,-	NP
8	15	29,30	-,-	NP
9	10	31*,32	-,-	-
9	15	33*,34	-,-	-
10	10	35*,36	-,-	-
10	15	37*,38	-,-	-
11	1	39,40*	-,-	-
11	10	41*,42	-,-	-
11	15	43*,44	-,-	-
12	10	45,46	-,-	NP
12	15	47,48	-,-	NP
13	10	49,50*	-,-	-
13	15	51,52	-,-	NP
14	10	53*,54*	-,-	-,-
14	15	55,56*	-,-	-
15	1	57*,58	-,-	-
15	10	59,60*	-,-	+
15	15	61*,62	-,-	+

\* These plates were tested in PCR. Results appear in PCR column.

by the addition of 0.05 ml of 50  $\mu\text{g/ml}$  propidium iodide (Sigma Chemical Co.) in phosphate-buffered saline (pH 7.4) to each well. After 24 to 48 hours, the pink/orange fluorescence-stained microplaques were visualized by placing the plates on an 8,000  $\mu\text{W/cm}^2$  304 nm UV light box (Fotodyne, Inc., New Berlin, Wis.). The plaques were counted at a magnification of 20x to 25x through a stereomicroscope.

TABLE 11

Sample	Log Titer
no QM	4.2
3 $\mu\text{g/ml}$ QM	3.4
10 $\mu\text{g/ml}$ QM	2.0
30 $\mu\text{g/ml}$ QM	<1.7

The results appear in Table 11, above. At a concentration of 30  $\mu\text{g/ml}$ , QM was able to inactivate cell-free HIV completely to the level of detection of the plaque assay used.

## EXAMPLE 8

The last example demonstrated that QM was able to inactivate cell free HIV. HIV can also be found within certain types of cells. This example examines the ability of QM, at varying concentrations, to inactivate the cell-associated form of HIV.

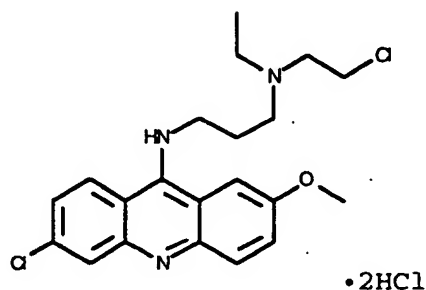
H9 cells chronically infected with HIV<sub>IIIB</sub> were used. (H9/HTLV-III-B NIH 1983 Cat.#400). Cultures of these cells were maintained in high glucose Dulbecco Modified Eagle Medium supplemented with 2 mM L-glutamine, 200 units/ml penicillin, 200  $\mu\text{g/ml}$  streptomycin, and 9% fetal bovine serum (Intergen Company, Purchase, N.Y.) For maintenance, the culture was split once a week, to a density of  $3 \times 10^5$  to  $4 \times 10^5$  cells/ml. About four days after splitting, 8.8% sodium bicarbonate was added as needed. For the inactivation procedure, the cells were used three days after they were split. They were spun from their culture medium at 400 g for 10 minutes, the supernatant was discarded, and the cells were resuspended in approximately 8 ml of 85% DMEM + 15% FBS, to a concentration of  $2 \times 10^6$  cells/ml. Aliquots (1 ml) of the infected cell suspension were placed in 15 ml tubes for QM free controls and for the QM experimental sample. A stock solution of QM (1 mg/ml in sterile ddH<sub>2</sub>O) was diluted into the

It is clear from this example that QM inactivates cell-associated HIV, even at very low concentrations such as 10µg/ml and below.

5

## EXAMPLE 9

This example sets forth the ability of two compounds having a nucleic acid binding ligand and a mustard group, QM, and N-(2-chloroethyl)-N-ethyl-N'-(6-chloro-2-methoxy-9-acridinyl)-1,3-propanediamine dihydrochloride ("ICR-170") (commercially available from Polysciences Inc, Warrington, PA) to inactivate  
10 both cell-free and cell-associated HIV in the presence of red blood cells. The structure of ICR-170 is shown below.



15 For the cell free HIV inactivation, 15 ml of PRBC was mixed with 5 ml Adsol for a final volume of 20 ml. Then ten 2 ml aliquots were added to 15 ml conical tubes. Varying doses of the two compounds were next added to the tubes. The stock compound solutions were both 1 mg/ml in saline, stored at 4 °C. ICR-170 was in solution at this concentration. The following volumes of the two test  
20 compounds were added to the PRBC tubes: 20, 40, 80 or 160 µl; to produce final concentrations of the test compound of 10, 20, 40, or 80 µg/ml.

After addition of the compounds, the samples were incubated for 100 minutes at room temperature in the dark, with mixing every 30 minutes. Subsequently, the red blood cells were pelleted by spinning for 5 minutes at 2500  
25 rpm. The supernatant was removed and NHSP was added so that the sample contained 15% NHSP. Samples were stored at -80°C.

Inactivation of cell-associated HIV was performed in a similar manner, with the following exceptions. H9 cells chronically infected with HIVIIIB were used. (H9/HTLV-III-B NIH 1983 Cat.#400). Cultures of these cells were  
30 maintained in high glucose DMEM supplemented with 2 mM L-glutamine, 200 units/mL penicillin, 200 µg/ml streptomycin, and 9% fetal bovine serum

TABLE 13B

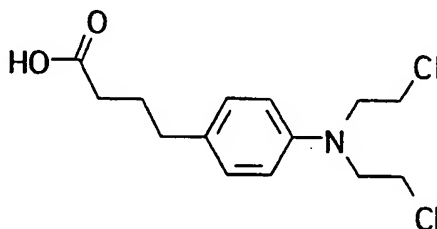
Compound	Concentration ( $\mu\text{g/ml}$ )	Log Titer	Log Reduction
QM	0	5.5	-
	10	4.4	1.1
	20	3.6	1.9
	40	2.5	3.0
	80	2.4	3.1
ICR-170	0	5.7	-
	10	5.2	0.5
	20	4.5	1.2
	40	3.7	2.0
	80	3.7	2.0

5

## EXAMPLE 10

The above examples have established that QM has exceptional pathogen inactivation activity. In choosing an agent to decontaminate blood products for clinical testing or transfusion, it is also important to consider the effects of the method and compound used on blood product function. This example explores the short term effects of two compounds, one having a nucleic acid binding ligand and a mustard group, QM and chlorambucil on red blood cell function, as measured by potassium leakage and IgG binding to red blood cell surfaces. The structure of chlorambucil appears below.

10



15

This example additionally compares the R17 inactivation activity, in red blood cells, of a compound having both a nucleic acid binding ligand and a mustard group (QM), with a compound having only a mustard group, and no nucleic acid binding ligand (chlorambucil).

20

Baxter Unival Anti-Human Globulin Anti-IgG for Direct Antiglobulin Test and Baxter Coombs control Cells for Quality Control of Anti-Human Globulin Test (both available from Baxter Healthcare Corporation, Deerfield, IL). The results of IgG Binding as measured by FACScan™ (Becton Dickinson, Mountain View, CA) appear in Table 16.

R17 was completely inactivated at all concentrations of QM ( $\geq 8.4$  logs/ml). However, little or no inactivation ( $\leq 0.4$  logs) was observed for Chlorambucil, up to a concentration of 300  $\mu\text{g/ml}$ .

TABLE 15

Sample	Extracellular Potassium (mM)						
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 6	Day 7
1	0.70	1.59	2.51	3.16	3.71	4.63	5.01
2	0.76	1.59	2.45	3.18	3.72	4.57	4.94
3	0.69	1.56	2.40	3.16	3.72	4.69	5.03
4	0.72	1.74	2.43	3.18	3.76	4.73	5.12
5	0.72	1.71	2.58	3.31	3.92	4.89	5.36
6	0.73	1.65	2.76	3.64	4.26	5.30	5.69
7	0.76	1.94	3.08	4.00	4.63	5.76	6.15
8	0.78	2.48	4.05	5.23	6.06	7.50	8.02
9	0.82	3.61	5.59	7.37	8.32	>10	11.20

Packed red blood cells may be stored for up to 42 days, depending upon the storage conditions, according to standards set by the United States Food and Drug Administration. During this storage time, the level of extracellular potassium increases. For example, red blood cells stored for 35 days in citrate phosphate dextrose adenine-1 (CPDA-1) have displayed potassium levels as high as 78.5 mM/L, an increase of 73.4 mM/L over the potassium level at day one of storage. *Immunohematology Principles and Practice*, p. 29, Table 2-1, J.B. Lippincott Company, Philadelphia, PA (1993). Units bearing such levels of extracellular potassium are transfused without breaching acceptable standards of practice, and are thus considered by the FDA to retain the ability to function for the purpose they are prepared and used.

cells were then gently resuspended and immediately examined for agglutination under microscope. All samples were negative for agglutination. Subsequently, one drop of Coombs positive control cells was added to the experimental samples. All samples then showed many medium and small agglutinates. The results of this experiment indicate that the treatment of red blood cells by the methods of the present invention did not interfere with the agglutination assays. These assay results were duplicated in a blinded study, using material from the same samples, by an independent laboratory.

Results of the above described experiment clearly confirm that the methods of the present invention do not stimulate IgG binding. The increase in fluorescence in Table 16 was the result of fluorescence from QM rather than a significant increase in IgG binding.

#### EXAMPLE 11

Example 10 showed that QM was able to inactivate R17 in red blood cells under conditions where potassium leakage and surface IgG binding were negligible. This example is designed to further these observations by looking more extensively at red blood cell function after treatment with varying levels of QM. Specifically, this example looks at the effects of QM treatment on red blood cell function after storage under conditions that closely mimic those in a blood bank.

A packed red blood cell unit, approximately 1 day old, was obtained from Sacramento Blood Center. The cells were resuspended and approximately 200 ml was transferred to a sterile container. R17 (0.2 ml) in LB was added and the sample was mixed. Next the unit was divided into 6-30 ml aliquots in sterile conical centrifuge tubes on ice. The remaining packed red blood cells were stored in the bag at 4°C.

QM (3.2 mg) was mixed with ice cold Adsol (1.6 ml) to make a 2.0 mg/ml suspension. Aliquots of the QM suspension were added to the cells as set forth in Table 17. The samples were mixed thoroughly by gentle inversion and transferred to Fenwal transfer packs (Baxter/Fenwal, Ill) for storage at 4°C.

TABLE 18B

QM Concentration	ATP (mM) Day 1	ATP (mM) Day 9	ATP (mM) Day 16
control	0.77	0.80	0.75
2.5 µg/ml	0.78	0.80	0.74
5 µg/ml	0.78	0.81	0.73
10 µg/ml	0.76	0.81	0.73
20 µg/ml	0.78	0.80	0.73
40 µg/ml	0.76	0.80	0.72

TABLE 18C

QM Concentration	2,3-DPG Day 1	2,3-DPG Day 9	2,3-DPG Day 16
control	2.20	0.77	0.89
2.5 µg/ml	2.20	0.88	0.10
5 µg/ml	2.35	0.97	0.28
10 µg/ml	2.06	1.11	0.34
20 µg/ml	2.63	1.43	1.36
40 µg/ml	2.09	1.04	0.11

TABLE 18D

QM Concentration	mean FL Day 1	median FL Day 1	mean FL Day 9	median FL Day 9	mean FL Day 16	median FL Day 16
control	4.41	4.1	4.41	4.1	4.41	4.1
2.5 µg/ml	4.77	4.45	4.77	4.45	4.77	4.45
5 µg/ml	4.79	4.45	4.79	4.45	4.79	4.45
10 µg/ml	4.96	4.7	4.96	4.7	4.96	4.7
20 µg/ml	5.73	5.19	5.73	5.19	5.73	5.19
40 µg/ml	6.31	6.04	6.31	6.04	6.31	6.04

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Under conditions of effective R17 inactivation in packed red blood cells, there are no significant effects on potassium-leakage, ATP content, 2,3-DPG content, or IgG binding to RBCs.

following dilutions of a 1.0 mg/ml solution: 150  $\mu$ l of a 1.0 mg/ml QM solution + 350  $\mu$ l DMSO to produce a 0.3 mg/ml solution; 40  $\mu$ l of a 1.0 mg/ml QM solution + 360  $\mu$ l DMSO to produce a 0.1 mg/ml solution; 15  $\mu$ l of a 1.0 mg/ml QM solution + 485  $\mu$ l DMSO to produce a 0.03 mg/ml solution. To the first tube, 100  $\mu$ l DMSO was added and the tube was placed on a 4°C shaker (25 rpm, Orbital Shaker, commercially available from VWR Scientific, Greenbelt, MO) for overnight incubation. Tubes 2 - 5 were shaken overnight as well, then 100  $\mu$ l aliquots of each QM solution was diluted into the tubes just before addition to the Ames strains. To tubes 6 - 9 was added 100  $\mu$ l of each QM solution. The tubes were then incubated overnight at 4°C on the shaker. Finally, 100  $\mu$ l of each QM solution was also added to tubes 10 - 13, which were then incubated on the shaker for 4 hours. Subsequently, 0.1 g of a polymeric adsorbent material, Amberlite XAD 16™ (commercially available from Sigma, Saint Louis, MO), was added to each of tubes 10 - 13 and the incubation was continued overnight. The final contents of each tube, and the stock QM solutions used, are listed in Table 19, below.

TABLE 19

SAMPLE NUMBER	CONTENTS	QM STOCK SOLUTION
1	RBC + DMSO	none
2	RBC + 0.003 mg/ml QM	0.03 mg/ml
3	RBC + 0.01 mg/ml QM	0.1 mg/ml
4	RBC + 0.03 mg/ml QM	0.3 mg/ml
5	RBC + 0.1 mg/ml QM	1 mg/ml
6	RBC + 0.003 mg/ml QM	0.03 mg/ml
7	RBC + 0.01 mg/ml QM	0.1 mg/ml
8	RBC + 0.03 mg/ml QM	0.3 mg/ml
9	RBC + 0.1 mg/ml QM	1 mg/ml
10	RBC + 0.003 mg/ml QM	0.03 mg/ml
11	RBC + 0.01 mg/ml QM	0.1 mg/ml
12	RBC + 0.03 mg/ml QM	0.3 mg/ml
13	RBC + 0.1 mg/ml QM	1 mg/ml

In a separate experiment, samples of QM in water were prepared as follows. Sample tubes were labeled and .5 ml phosphate buffer was added to each one. Then various dilutions of a stock solution of QM (1 mg/ml) were added to five

incubation growth was only seen on the side of the plate shielded from UV irradiation.

*R-factor:* The tester strains (TA97a, TA98, TA100, and TA102) contain the pKM101 plasmid that increases their sensitivity to mutagens. The plasmid also confers resistance to ampicillin to the bacteria. This was confirmed by growing the strains in the presence of ampicillin.

*pAQ1:* Strain TA102 also contains the pAQ1 plasmid that further enhances its sensitivity to mutagens. This plasmid also codes for tetracycline resistance. To test for the presence of this plasmid TA102 was streaked on a minimal glucose plate containing histidine, biotin, and tetracycline. The plate was incubated for 16 hours at 37°C. The strain showed normal growth indicating the presence of the pAQ1 plasmid.

The same cultures used for the genotype testing were again cultured and aliquots were frozen under controlled conditions. The cultures were again tested for genotype to confirm the fidelity of the genotype upon manipulation in preparing the frozen permanents.

The first tests done with the strains were to determine the range of spontaneous reversion for each of the strains. With each mutagenicity experiment the spontaneous reversion of the tester strains to histidine independence was measured and expressed as the number of spontaneous revertants per plate. This served as the background controls. A positive mutagenesis control was included for each tester strain by using a diagnostic mutagen suitable for that strain (2-aminofluorene at 5 mg/plate for TA98; sodium azide at 1.5 mg/plate for TA100; 9-aminoacridine for TA 1537).

For all experiments, the pre-incubation procedure was used. In this procedure one vial of each tester strain was thawed and tubes were prepared for each strain, containing 20 µL of the culture and 6 mL of Oxoid Nutrient Broth #2. This solution was allowed to shake for 10 hours at 37°C. In the pre-incubation procedure, for each tester strain used to evaluate the test solution, 0.1 mL of the overnight culture was added to each of 13 sterile test tubes. To each of the tubes, 0.1 mL of the test solution from tubes 1 - 13 was added. This was also performed on the samples containing QM in water only. Then 0.5 mL of 0.2 M sodium phosphate buffer, pH 7.4 was added. The 0.7 mL mixture was vortexed and then pre-incubated while shaking for 20 minutes at 37°C. After shaking, 2 mL of molten top agar supplemented with histidine and biotin were added to the 0.7 mL mixture and immediately poured onto a minimal glucose agar plate

TABLE 20

TEST	100 $\mu$ l saline	400 $\mu$ l saline	20 $\mu$ g/ml QM	40 $\mu$ g/ml QM	60 $\mu$ g/ml QM	20 $\mu$ g/ml ICR170	40 $\mu$ g/ml ICR170	60 $\mu$ g/ml ICR170
Glucose	95	92	93	93	90	94	92	90
BUN	17	17	17	17	17	17	17	17
Creatinine	1.1	1.1	1.1	1.2	1.1	1	1.1	1.1
Bun/Creat. ratio	15	15	15	14	15	17	15	15
Sodium	143	143	138	144	146	143	144	144
Potassium	4	3.9	4	3.9	3.9	4	4.1	4.1
Chloride	104	106	104	106	107	103	104	103
Magnesium	1.7	1.6	1.6	1.6	1.6	1.7	1.6	1.5
Calcium	9.3	8.8	9.3	8.8	9	9.4	9.2	9.1
Phos- phorous inorganic	4.3	3.9	4.1	4.1	4.1	4.3	4.3	4.1
protein, total	7.3	7.1	7.4	7.3	7.1	7.3	7.3	7.1
albumin	4.6	4.5	4.6	4.6	4.4	4.6	4.6	4.4
globulin, total	2.7	2.6	2.8	2.7	2.7	2.7	2.7	2.7
A/G ratio	1.7	1.7	1.6	1.7	1.6	1.7	1.7	1.6
billirubin	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
alk	55	50	48	50	51	54	51	47
LDH	136	134	121	125	138	129	138	151
GGT	23	21	22	22	22	23	20	16
AST	17	16	18	16	16	16	17	16
ALT	20	19	17	19	18	19	19	19
Uric Acid	5.6	5.3	5.5	5.4	5.4	5.7	5.6	5.5
Iron	119	107	117	113	115	119	122	120
Triglyceride	164	152	159	158	158	161	150	156
Cholesterol	234	221	227	226	223	231	231	223

The supernatant was removed and saved as "washed solution". The red blood cell pellet was resuspended in Adsol to make a 50% Htc solution. Three 1.5 ml aliquots were transferred to 14 ml round bottom polypropylene tubes.

Next, 1 mg/ml solutions of two compounds, QM and ICR-170, were prepared in saline. The compounds were added to the three polypropylene tubes as follows: tube 1 received no treatment (120 µl of saline was added); tube 2 received 120 µl of the 1 mg/ml solution of QM for a final concentration of 80 µg/ml; tube 3 120 µl of the 1 mg/ml solution of ICR-170, for a final concentration of 80 µg/ml.

The samples were then incubated for 2 hours at 4° C. Cells were washed three times, each time by adding 6 ml Adsol to each tube and spinning the samples at 1800 rpm for 5 minutes. After the final wash, the pellet was resuspended in Adsol buffer to a concentration of  $4 \times 10^6$  cells/µl. 50 µl of each sample was removed for an unstained control.

The remaining cells were then stained with PKH26 dye. From a 1mM PKH26 stock, 120 µl was removed and diluted with 8 ml diluent A to create a working solution of 15.7 µM PKH26. This solution was stored in the dark until use. To each 2 ml of cells, 2 ml of PKH26 dye was added. The samples were mixed gently and incubated for 5 minutes at room temperature in the dark. The cells were remixed after 2.5 minutes. After another 5 minute incubation, 2 volumes of the reserved "washed solution" was added to stop the staining reaction. The cells were centrifuged at 1800 rpm for 5 minutes to pellet and the supernatant was removed. The cells were then washed 3 times with Adsol buffer, as before. After the final wash, the sample volume was restored to 1 ml with Adsol. An aliquot of each sample was removed at this point for a positive stained control sample and counted on the Sysmex machine.

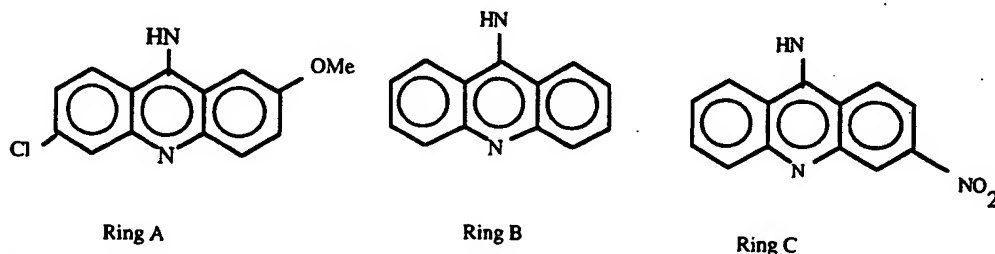
Swiss Webster mice were transfused with 0.2 ml of the labeled cells from each of samples 1-3 via tail vein injection. The mice were then weighed to calculate the blood volume. Blood volume is calculated as the animal weight in gm  $\times$  0.06. Then, blood was drawn from the mice by retroorbital venipuncture using heparin-EDTA coated capillary tubes at 1 hour, 24 hours, 2 more times during the first week, and one time weekly for 3 weeks. The eye bleeding samples were drained into isotonic solution before analysis.

Samples were analyzed on a FACScan™ at the FL2 (red fluorescent channel) with gating on the red cell population using forward and side scatter

## EXAMPLE 16

This example sets forth the results of several screens for viral inactivation performed on compounds of the present invention which have acridine groups as their nucleic acid binding ligands. Descriptions of how these screens were performed are found in the above examples discussing R-17, cell free HIV and cell associated HIV. Table 23, below, shows results in terms of the anti-viral activity of nucleic acid specific alkylating agents comprising: acridine ring - linking chain -  $N(CH_2CH_2Cl)_2$ . Activity is set forth by + for lowest, up to ++++ for complete inactivation (to the level of detection used).

TABLE 23



Cmpd	Ring	linker	R17a	CA-HIV <sup>b</sup>	CF-HIV <sup>b</sup>
2	A	(CH <sub>2</sub> ) <sub>2</sub>	+		
3	A	(CH <sub>2</sub> ) <sub>3</sub>	++	+	+
4	B	(CH <sub>2</sub> ) <sub>3</sub>		+	+
5	A	(CH <sub>2</sub> ) <sub>4</sub>	++++	++++	+++
1	A	CH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub>	++++	+++	+++
6	A	(CH <sub>2</sub> ) <sub>5</sub>	++++	++++	+++
7	B	(CH <sub>2</sub> ) <sub>5</sub>		++++	++++
8	C	(CH <sub>2</sub> ) <sub>5</sub>		+	++
9	A	(CH <sub>2</sub> ) <sub>6</sub>	++++	+++	++
10	B	(CH <sub>2</sub> ) <sub>6</sub>		++++	+++
11	A	CH <sub>2</sub> CONH(CH <sub>2</sub> ) <sub>3</sub>	++++	+++	++
12	A	CH(CH <sub>3</sub> )CONH(CH <sub>2</sub> ) <sub>2</sub>	++++	+++	+
13	A	CH(CH <sub>3</sub> )CONH(CH <sub>2</sub> ) <sub>3</sub>	++++	+++	+

<sup>a</sup> ++++ shows  $\geq 6$  log reduction at 5uM compound; <sup>b</sup> ++++ shows  $\geq 4$  log reduction at 20  $\mu$ M.

4 times, each time with 20 mL of  $\text{CHCl}_3$ . This last organic solution was rinsed with 2 x 20 mL of brine, dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated to give 0.43 g (79%) of the aminediol, 5-[N,N-bis-(2-hydroxyethyl)amino]methyl-8-methoxypsoralen, melting point 121-122 °C; NMR ( $\text{CDCl}_3$ ): 2.71 (t, J = 5 Hz, 4H), 3.61 (t, J = 5 Hz, 4H), 4.09 (s, 2H), 4.29 (s, 3H), 6.41 (d, J = 10 Hz, 1H), 7.00 (d, J = 2 Hz, 1H), 7.70 (d, J = 2 Hz, 1H), 8.38 (d, J = 10 Hz, 1H).

Step 3: 5-[N,N-Bis(2-chloroethyl)amino]methyl-8-methoxypsoralen hydrochloride.

5-[N,N-Bis(2-hydroxyethyl)amino]methyl-8-methoxypsoralen (0.030 g, 0.090 mmol) was dissolved in 1 ml thionyl chloride. It was covered with a serum cap with a small needle vent and allowed to stir for 3 days. The reaction mixture was stripped and the crude solid was recrystallized in isopropanol to give 5-[N,N-bis(2-chloroethyl)amino]methyl-8-methoxypsoralen hydrochloride (0.012 g, 32.4%) as an off-white solid, mp 158-162 °C.  $^1\text{H}$ NMR ( $\text{CD}_3\text{OD}$ ): 3.40 (t, J = 6Hz, 4H), 3.86 (t, J = 6Hz, 4H), 4.33 (s, 3H), 4.70 (s, 2H), 6.52 (d, J = 10Hz, 1H), 7.28 (d, J = 2Hz, 1H), 8.03 (d, J = 2Hz, 1H), 8.48 (d, J = 10Hz, 1H). The chemical shift appeared to be sensitive to trace acid present.

A portion of the above salt was partitioned between methylene chloride and aqueous  $\text{NaHCO}_3$ . The organic layer was again washed with aqueous bicarbonate, dried with brine, then dried with anhydrous  $\text{Na}_2\text{SO}_4$  and evaporated to give the neutral amine; mass spectrum (EI, m/e): 371 (3), 369 (4), 230 (16), 229 (100), 214 (5), 201 (5), 186 (10) (obtained on a Shimadzu QP5000 GC/MS, with Rtx-5, 15m column, commercially available from Shimadzu Corporation, Kyoto, Japan).

#### EXAMPLE 18

This example describes a contemplated embodiment wherein red blood cells are treated by a method of the present invention. The standard blood product separation approach used presently in blood banks is as follows: three bags are integrated by flexible tubing to create a blood transfer set (e.g., commercially available from Baxter, Deerfield, Ill.). After blood is drawn into the first bag, the entire set is processed by centrifugation (e.g., Sorvall<sup>TM</sup> swing bucket centrifuge, Dupont), resulting in packed red cells and platelet rich plasma in the first bag. The plasma is expressed off of the first bag (e.g., using a

off the solvent, the resulting syrup was dissolved in  $\text{CH}_2\text{Cl}_2$ , washed with water several times, then brine, dried with anhydrous  $\text{Na}_2\text{SO}_4$  and stripped of solvent to give 8-[3-(bis-2-hydroxyethyl)amino]propyloxypsoralen (0.499 g, 92.9% yield), as a brown syrup.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): d 2.04 (quintet,  $J = 6.2$  Hz, 2H), 2.72 (t,  $J = 5.2$  Hz, 4H), 2.91 (t,  $J = 6.6$  Hz, 2H), 3.70 (t,  $J = 5.3$  Hz, 4H), 4.54 (t,  $J = 5.7$  Hz, 2H), 6.38 (d,  $J = 9.6$  Hz, 1H), 6.82 (d,  $J = 2.1$  Hz, 1H), 7.39 (s, 1H), 7.70 (d,  $J = 2.1$  Hz, 1H), 7.79 (d,  $J = 9.5$  Hz, 1H).

STEP 3: 8-[3-(Bis-2-chloroethyl)amino]propyloxypsoralen, (compound 5)  
Thionyl chloride (0.030 mL, 0.41 mmol) was added dropwise to an ice bath chilled solution of 8-[3-(Bis-2-hydroxyethyl)amino]propyloxypsoralen (20.0 mg, 0.0575 mmol) and pyridine (0.016 mL, 0.21 mmol) in benzene (2 mL) and  $\text{CH}_2\text{Cl}_2$  (2 mL). The reaction mix was covered with a serum cap and allowed to stir overnight at room temperature, then stripped under reduced vacuum and partitioned between  $\text{CH}_2\text{Cl}_2$  and 10 %  $\text{NaHCO}_3$ . The organic layer was washed several times with aqueous  $\text{NaHCO}_3$ , then brine. After drying with anhydrous  $\text{Na}_2\text{SO}_4$ , solvent was rotovapped off to give 8-[3-(bis-2-chloroethyl)amino]propyloxypsoralen (14.0 mg, 57.8 % yield), as a yellow solid.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): d 1.97 (app quintet  $J = 6.3$  Hz, 2H), 2.91 (app t,  $J = 7.5$  Hz, 6H), 3.55 (t,  $J = 7.0$  Hz, 4H), 4.58 (t,  $J = 5.9$  Hz, 2H), 6.37 (d,  $J = 9.5$  Hz, 1H), 6.82 (d,  $J = 2.1$  Hz, 1H), 7.37 (s, 1H), 7.70 (d,  $J = 2.1$  Hz, 1H), 7.77 (d,  $J = 9.5$  Hz, 1H).

8-[5-(Bis-2-chloroethyl)amino]pentyloxypsoralen (compound 6)  
In the same manner as the foregoing, but using 1,5-dibromopentane in Step 1 in place of 1,3-dibromopropane, compound 6 is produced.

## EXAMPLE 20

This example describes the synthesis of 5-[3-(Bis-2-chloroethyl)aminopropoxy]methyl-8-methoxypsoralen, (Compound 4)

STEP 1: 5-[(3-Hydroxy)-propyloxy]methyl-8-methoxypsoralen  
5-Bromomethyl-8-methoxypsoralen<sup>®</sup> (0.300 g, 0.970 mmol) and 1,3-propanediol (4 mL, 55.3 mmol) were refluxed in acetone (30 mL) for 3 days. The solvent was removed under reduced pressure and the residue was dissolved in  $\text{CH}_2\text{Cl}_2$  and washed several times with water to remove excess diol. After

vacuum and partitioned between  $\text{CH}_2\text{Cl}_2$  and 10 %  $\text{NaHCO}_3$ . The organic layer was washed several times with aqueous  $\text{NaHCO}_3$ , then brine. After drying with anhydrous  $\text{Na}_2\text{SO}_4$ , solvent was rotovapped off to give 5-[3-(bis-2-chloroethyl)aminopropoxy]methyl-8-methoxypsoralen (0.0075 mg, 63.0 % yield), as a syrup.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): d 1.71 (t, J = 6.1 Hz, 2H), 2.60 (t, J = 6.4 Hz, 2H), 2.82 (t, J = 6.6 Hz, 4H), 3.3-3.7 (m, 6H), 4.29 (s, 3H), 4.86 (s, 2H), 6.43 (d, J = 9.9 Hz, 1H), 6.96 (s, 1H), 7.71 (s, 1H), 8.15 (d, J = 9.9 Hz, 1H).

#### EXAMPLE 21

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This example describes the synthesis of 5-[3-(Bis-2-chloroethyl)aminopropoxy]methyl-8-methoxypsoralen, (compound 16)

STEP 1: 5'-[4-(Bis-2-hydroxyethyl)amino-1-butylaminomethyl]-4,4',8-trimethylpsoralen

A solution of 5'-bromomethyl-4,4',8-trimethylpsoralen (US Patent 4,294,822, 73.0 mg, 0.227 mmol), and N,N-bis(2-hydroxyethyl)-1,4-butanediamine (400 mg, 2.27 mmol) were stirred in acetonitrile (100 mL) for 4 h. After rotovapping off the solvent, the resulting syrup was dissolved in  $\text{CH}_2\text{Cl}_2$ , washed with 0.3M HCl several times, then chilled in an ice/water bath and made basic with powdered  $\text{K}_2\text{CO}_3$ . The product was extracted from the aqueous layer with several portions of  $\text{CH}_2\text{Cl}_2$ . The organic solution was rinsed with brine then dried with anhydrous  $\text{Na}_2\text{SO}_4$  and stripped to give a brown syrup. The crude product was chromatographed by TLC (silica gel, 1/9 MeOH/ $\text{CHCl}_3$ ) dissolved in  $\text{CH}_2\text{Cl}_2$  to give 5'-[4-(bis-2-hydroxyethyl)amino-1-butylaminomethyl]-4,4',8-trimethylpsoralen, as a yellow syrup (26.9 mg, 29 % yield).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): d 1.59 (s, 3H), 2.49-2.76 (m, 14H), 3.62 (t, J = 5.3 Hz, 4H), 3.97 (s, 2H), 6.23 (d, J = 1.1 Hz, 1H), 7.47 (s, 1H).

STEP 2: 5'-[4-(Bis-2-chloroethyl)amino-1-butylaminomethyl]-4,4',8-trimethylpsoralen

Thionyl chloride (0.020 mL, 0.278 mmol) was added dropwise to an ice bath chilled solution of 5'-[3-(bis-2-hydroxyethyl)amino-1-butylaminomethyl]-4,4',8-trimethylpsoralen (11.6 mg, 0.0278 mmol) and pyridine (0.023 mL, 0.278 mmol) in  $\text{CH}_2\text{Cl}_2$  (5 mL). The reaction mix was covered with a serum cap and allowed to stir overnight at room temperature, then stripped under reduced

4'-[3-(Bis-2-chloroethyl)amino-1-hexylaminomethyl]-4,5',8-trimethylpsoralen, (compound 13)

In the same manner as the foregoing, but using 4'-bromomethyl-4,5',8-trimethylpsoralen and N,N-bis(2-hydroxyethyl)-1,6-hexanediamine in Step 1 in place of 5'-bromomethyl-4,4',8-trimethylpsoralen and N,N-bis(2-hydroxyethyl)-1,4-butanediamine respectively, the title compound is produced.

## EXAMPLE 22

This example describes the synthesis of 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, (compound 9)

STEP 1: 4'-[(4-Methanesulfonyl)-butoxy]methyl-4,5',8-trimethylpsoralen

A solution of 4'-[(4-hydroxy)-butoxy]methyl-4,5',8-trimethylpsoralen (1-U.S. Patent 4,269,852, 91.5 mg, 0.301 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) was chilled with an ice/water bath. Triethylamine (0.14 mL, 1.00 mmol) then methanesulfonyl chloride (0.070 mL, 0.903 mmol) were added dropwise. The solution was allowed to warm to room temperature and stirred overnight. The reaction mixture was partitioned between CH<sub>2</sub>Cl<sub>2</sub> and water. The organic layer was washed several times with aqueous NaHCO<sub>3</sub>, then brine, and dried with anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated to give crude 4'-[(4-methanesulfonyl)-butoxy]methyl-4,5',8-trimethylpsoralen (0.106 g, 86.2 % crude yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 1.71-1.86 (m, 4H), 2.47 (s, 6H), 2.52 (s, 3H), 2.95 (s, 3H), 3.51 (t, J = 6.0, 2H), 4.21 (t, J = 6.2, 2H), 4.59 (s, 2H), 6.20 (s, 1H), 7.54 (s, 1H).

STEP 2: 4'-[4-(Bis-2-hydroxyethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen

A solution of crude 4'-[(4-methanesulfonyl)-butoxy]methyl-4,5',8-trimethylpsoralen (106 mg, 0.260 mmol) and diethanolamine (300 mg, 2.85 mmol) were refluxed in acetonitrile (8 mL) overnight. After rotovapping off the solvent, the resulting syrup was dissolved in CH<sub>2</sub>Cl<sub>2</sub>, washed several times with aqueous NaHCO<sub>3</sub>, then brine, and dried with anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated to give crude product which was chromatographed by TLC (silica gel, 95/5 CHCl<sub>3</sub>-MeOH) to give a 4'-[4-(bis-2-hydroxyethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen as a yellow solid (32 mg, 27.1 % yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>):

TABLE 24

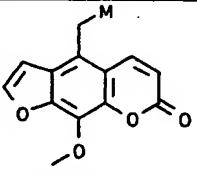
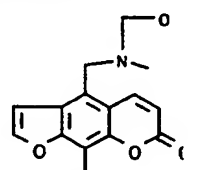
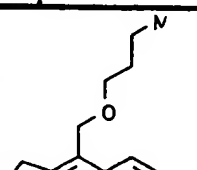
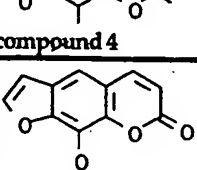
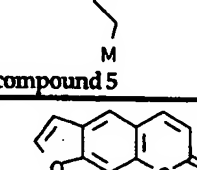
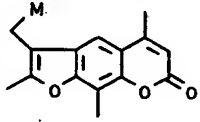
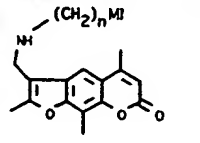
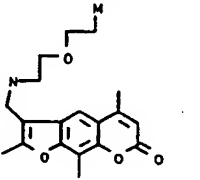
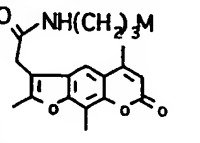
Compound	Dose for 5 log kill ( $\mu$ M)	Logs Virus Killed at Fixed Drug Dose			
		R-17 0.5 $\mu$ M	R-17 2 $\mu$ M	R-17 5 $\mu$ M	CA HIV 15 $\mu$ M
M = N(CH <sub>2</sub> CH <sub>2</sub> Cl) <sub>2</sub> NT = not tested					
QM	$\leq 0.5$	>6	>6	>6	2.8-4.2
 compound 2	>30	<0.5	<0.5	0.5	NT
 compound 3	>75	<0.5	<0.5	<0.5	NT
 compound 4	12-25	<0.5	0.5	2-3	NT
 compound 5	10	<0.5	<0.5	2-3	NT
 compound 6	>30	<0.5	<0.5	1	NT

TABLE 26

Compound	Dose for 5 log kill ( $\mu$ M)	Logs Virus Killed at Fixed Drug Dose			
		R-17 0.5 $\mu$ M	R-17 2 $\mu$ M	R-17 5 $\mu$ M	CA- HIV 15 $\mu$ M
$M = N(CH_2CH_2Cl)_2$ NT = not tested	R-17	R-17 0.5 $\mu$ M	R-17 2 $\mu$ M	R-17 5 $\mu$ M	CA- HIV 15 $\mu$ M
 compound 10	>30	NT	0.5	NT	NT
 n=3 compound 11	$\leq 2$	1	6	>6	0.4
n=4 compound 12	NT	NT	NT	NT	0.8
n=6 compound 13	$\leq 2$	NT	>6	>6	1.5
 compound 14	NT	NT	NT	NT	1.0
 n=3 compound 15	NT	NT	NT	NT	0.3

## CLAIMS

We claim:

1. A method of inactivating pathogens in a blood product, comprising:
  - a) adding a compound having a mustard group and a nucleic acid binding ligand, selected from the group comprising a psoralen group and an acridine group, to a blood product suspected of containing pathogens and mixing to create a mixture, said compound reaching a final concentration sufficient to inactivate substantially all of said pathogens, and
  - b) incubating said mixture without significant damage to said blood product, to create an incubated mixture.
2. The method of Claim 1, wherein said compound is added to said blood product to a final concentration of said compound of between 1 µg/ml and 250 µg/ml.
3. The method of Claim 1, wherein said mixture is incubated for between 1 minute and 48 hours.
4. The method of Claim 1, wherein said mixture is incubated for between approximately 12 and 24 hours.
5. The method of Claim 1, wherein when said compound is added to said blood product, said compound is in a mixture comprising dextrose, sodium chloride, mannitol, adenine and H<sub>2</sub>O.
6. The method of Claim 1, further comprising: c) transfusing said incubated mixture into a mammal.
7. The method of Claim 1, wherein said blood product comprises red blood cells.
8. The method of Claim 1, further comprising c) washing said blood product to remove compound from said incubated mixture.

17. The method of Claim 14, further comprising: c) transfusing said incubated mixture into a mammal.

18. The method of Claim 14, wherein said pathogens comprise viral  
5 pathogens.

19. The method of Claim 14, wherein said pathogens comprise bacterial pathogens.

20. The method of Claim 14, further comprising, (c) washing said blood product to remove compound from said incubated mixture.

21. The method of Claim 14, wherein said compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxypsoralen, 5-[3-  
15 (Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxypsoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxypsoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine.

22. The method of Claim 14, wherein more than one of said compounds is added to said blood product.

23. The method of Claim 14, further comprising: c) after incubating said mixture, removing said compound from said incubated mixture with an  
25 adsorbent material.

24. An improved method of treating biological compositions, comprising:

- 30 a) adding a compound having a nucleic acid binding ligand and a mustard group to a biological composition and mixing to create a mixture, and  
b) incubating said mixture to create an incubated mixture.

25. The method of Claim 24, wherein said compound is added to said biological composition to a final concentration of said compound of between  
35 1µg/ml and 250 µg/ml.

36. An improved method of treating clinical samples, comprising, in the following order:

a) providing: (1) a compound having a mustard group and a nucleic acid binding ligand, selected from the group consisting of a psoralen group and an acridine group, and (2) a clinical sample intended for in vitro clinical testing;

b) adding said compound to said clinical sample and mixing to create a mixture,

c) incubating said mixture for between 1 minute and 48 hours, and

d) measuring the level of a clinical chemistry analyte in said clinical sample.

37. The method of Claim 36, wherein said compound is selected from the group consisting of: 8-[3-(Bis-2-chloroethyl) amino]propyloxypsoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxypsoralen, 5-[3-(Bis-2-chloroethyl) aminopropoxy]methyl-8-methoxypsoralen, 4'-[4-(Bis-2-chloroethyl)aminobutoxy]methyl-4,5',8-trimethylpsoralen, and N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine.

38. The method of Claim 36, wherein said clinical sample comprises red blood cells.

39. The method of Claim 38, wherein said red blood cells further comprise viral pathogens.

40. The method of Claim 38, wherein said red blood cells further comprise bacterial pathogens.

41. The method of Claim 36, wherein step c) is performed without significant damage to said clinical chemistry analog.

42. A method of inactivating pathogens in a red blood cell containing composition, comprising:

a) adding a compound having a nucleic acid binding ligand and a mustard group to a blood product comprising red blood cells, where said blood product is suspected of containing pathogens, to create a mixture, said compound

50. The method of Claim 42, wherein said nucleic acid binding ligand of said compound is selected from the group comprising: a psoralen group and a 9-aminoacridine group.

5 51. A method of inactivating pathogens in a red blood cell containing composition, comprising:

a) adding a compound having an acridine group and a mustard group to a blood product comprising red blood cells, where said blood product is suspected of containing pathogens, to create a mixture, said compound reaching  
10 a final concentration sufficient to inactivate substantially all of said pathogens, and

b) incubating said mixture in vitro for between 1 minute and 48 hours, while retaining the ability of said blood product to function for the purpose said blood product was prepared, to create an incubated mixture.  
15

52. The method of Claim 51, wherein said compound is added to said blood product comprising red blood cells to a final concentration of between 1µg/ml and 250 µg/ml.

20 53. The method of Claim 51, wherein when said compound is added to said blood product comprising red blood cells, said mixture further comprises dextrose, sodium chloride, mannitol, adenine and H<sub>2</sub>O.

54. The method of Claim 51, further comprising: c) transfusing said  
25 mixture into a mammal.

55. The method of Claim 51, wherein said pathogens comprise viral pathogens.

30 56. The method of Claim 51, wherein said pathogens comprise bacterial pathogens.

57. The method of Claim 51, wherein said compound is N1,N1-bis (2-chloroethyl)-N4-(6-chloro-2-methoxy-9-acridinyl)-1,4-pentanediamine.  
35

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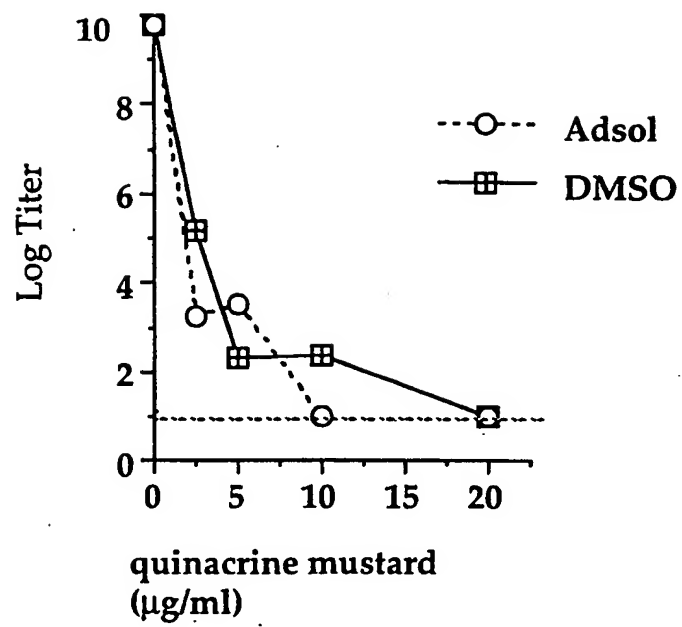


FIG. 1

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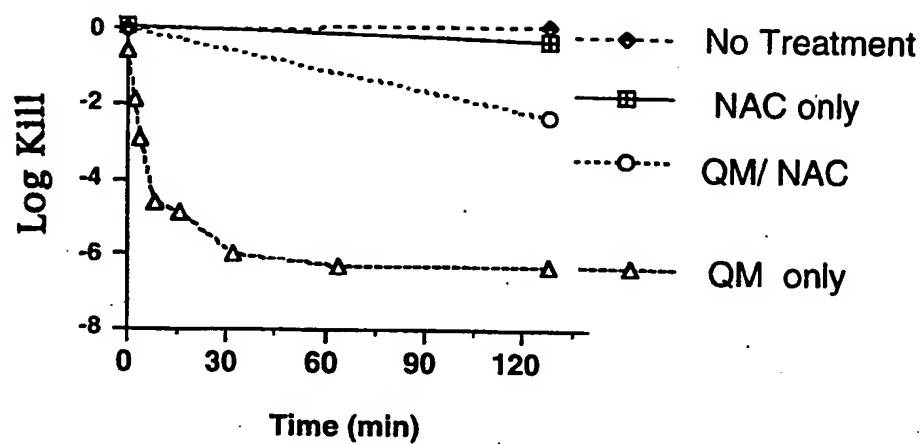


FIG. 3

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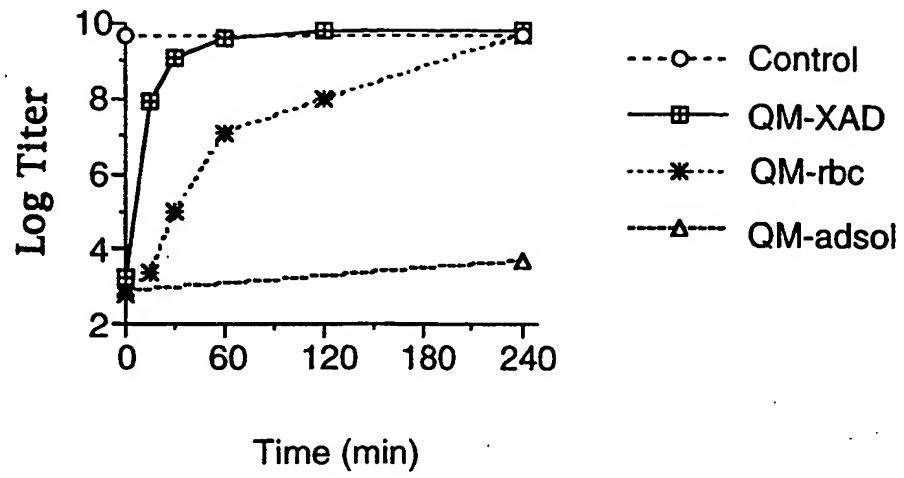


FIG. 5

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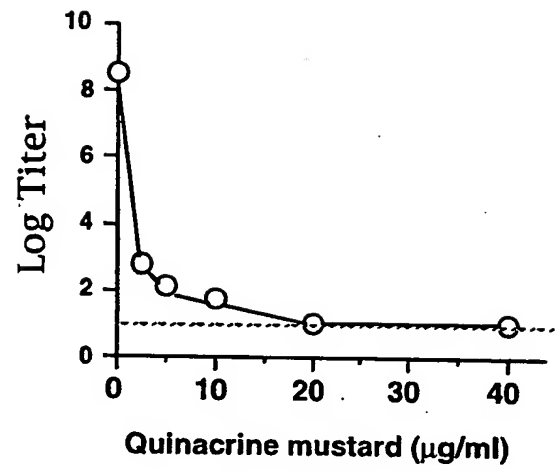


FIG. 7

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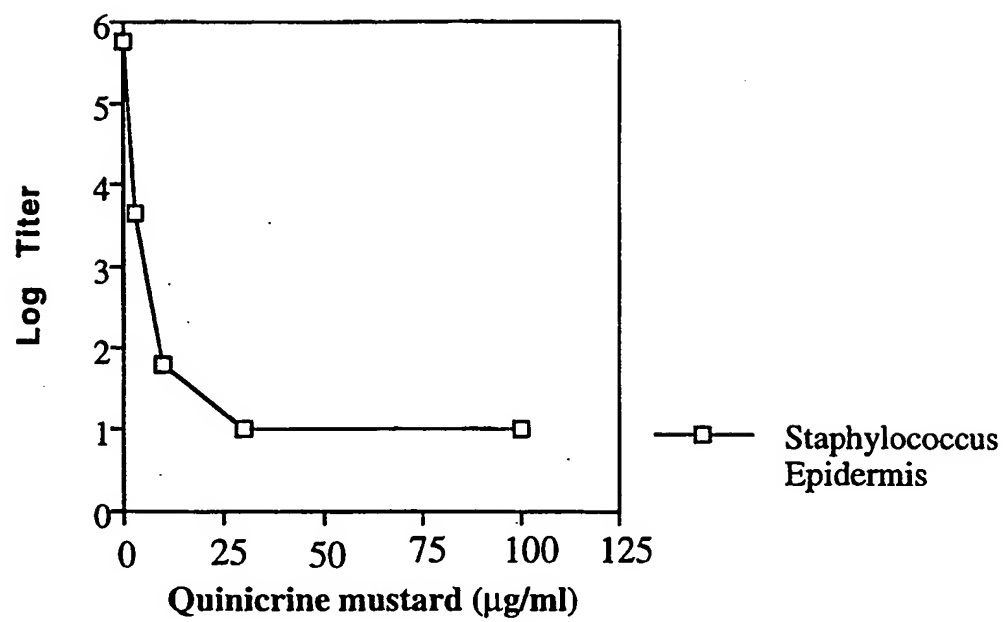


FIG. 9

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/09616

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☒ No protest accompanied the payment of additional search fees.